Selective alcohol oxidations with artificial oxidases: alcohol dehydrogenase – NADPH oxidase fusions

Friso S. Aalbers and Marco W. Fraaije
Molecular Enzymology Group
University of Groningen, The Netherlands

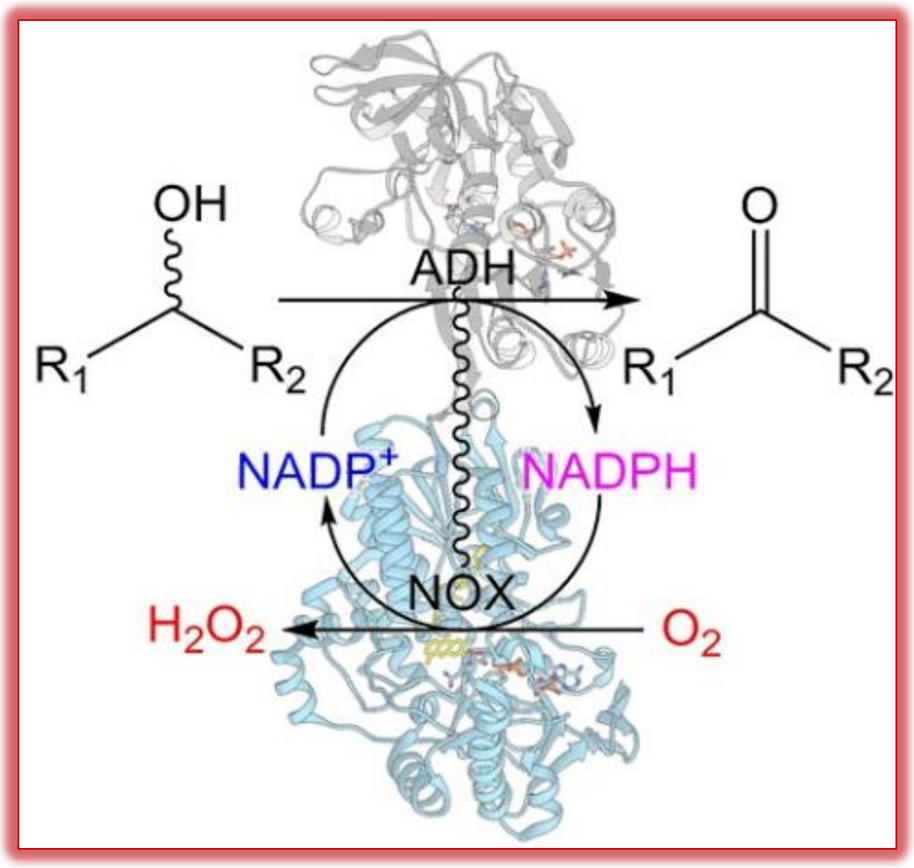
ABSTRACT

Selective alcohol oxidations are useful for production of ketones/aldehydes, and for kinetic resolutions of racemic alcohols.[1] Alcohol oxidases (AOXs) and Alcohol Dehydrogenases (ADHs) can catalyze oxidations. Not many AOXs are available, compared to ADHs. ADHs rely on NAD(P)$^+$ for oxidations, and oxidations are thermodynamically less favored – efficient recycling is needed.[1]

Fusion enzymes of three ADHs with an NADPH oxidase (NOX) were produced and characterized (ADHs: *Lb*RADH, *Tb*SADH, ADHA). The fusion enzymes were successfully applied for conversions, and for two activity screening methods: cell extract-based, colony-based.

RESULTS

The fusion enzymes showed good expression with 40-120 mg/L culture (pBAD in *E. coli*). The activity & kinetics were similar to the non-fused enzymes. Conversions were greatly improved by including FAD and catalase.



OUTCOME

1. High total turnover numbers (TTN) could be attained for both enzyme and cofactor NADP$^+$. Kinetic resolution of *rac*-1-phenyl ethanol could be performed using the NOX-ADH fusions, with high enantiomeric excess.

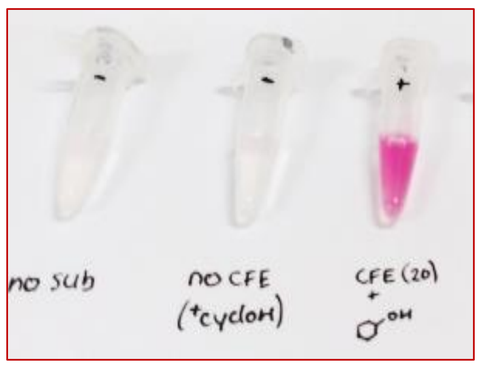
2. Cell extract could be used for rapid activity assay using horseradish peroxidase, without addition of NADP$^+$/NADPH.

3. Colonies expressing NOX-ADH fusion could be screened for alcohol oxidation activity – active colonies turned dark purple [2].

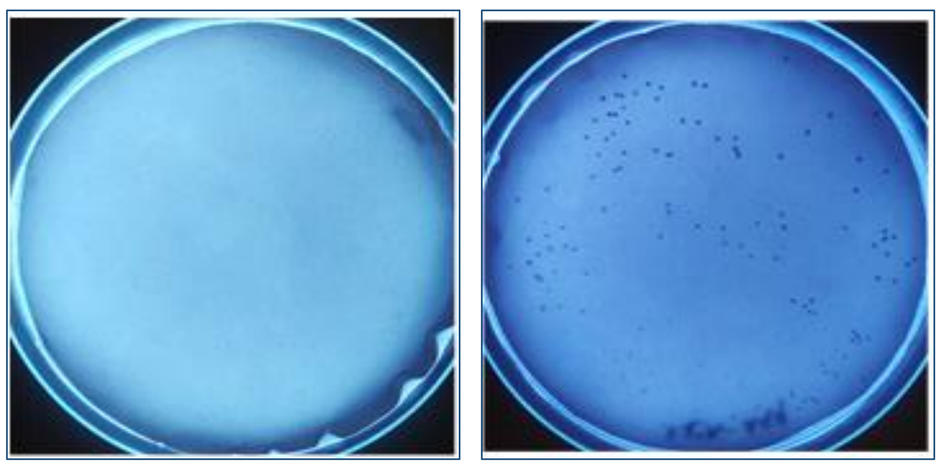
1. Conversions isolated fusion enzymes

Enzyme	Substrate	*X* (%)	ee	TTN (enzyme)	TTN (cofactor)
NOX-A	cyclohexanol	95 %	n.a.	31 666	475
NOX-L	cyclohexanol	99 %	n.a.	33 000	495
T-NOX	cyclohexanol	69 %	n.a.	23 000	345
NOX-A	rac-1-phenylethanol	94 %	99% (*R*)	31 333	470
*NOX-A	rac-1-phenylethanol	56 %	99% (*R*)	28 000	140
NOX-L	rac-1-phenylethanol	50 %	99% (*S*)	16 666	250

2. Conversions with cell extracts



3. Facile screening assay with fusion enzymes



CONCLUSIONS

- Expression-level, activity and enantioselectivity were retained.
- The ADH-NOX fusions could be applied as 'artificial oxidases': catalyzing continous alcohol oxidations using only dioxygen, reaching high TTNs.
- With the NOX fusion partner, cell extracts can be used to rapidly measure alcohol oxidation activity through a HRP-coupled assay.
- Colonies expressing ADH-NOX fusion can be screened for specific alcohol oxidation activity – useful tool for screening of large mutant libraries.

[1] G. Rehn, A. T. Pedersen, J. M. Woodley, *J. Mol. Catal. B Enzym.* 2016, 134, 331–339.
[2] M. Alexeeva, A. Enright, M. J. Dawson, M. Mahmoudian, N. J. Turner, *Angew. Chemie* 2002, 114, 3309–3312.
[3] F. S. Aalbers, M. W. Fraaije, submitted, 2018

Conditions

1. Reaction conditions: 5 µM enzyme fusion, 50 mM substrate, 100 µM NADP$^+$, 10 µM FAD, 1 kU catalase, 50 mM Tris/HCl pH 8.5, 0.5 mL volume, 24 °C, 64 hours. *For 24 hours.

2. The reaction included: 50 mM Tris/HCl pH 7.5, HRP (0.8 U), AAP (0.1 mM) and DCHBS (1 mM), cell-free extract containing NOX-A (10 % v/v) and 30 mM cyclohexanol. Controls: no substrate, and no cell-free extract.

3. Method based on [2]. Transformed cells plated onto LB agar plate with 0.2% arabinose and a porous membrane, incubated for 40 hours at 24 °C. Removed membrane with colonies, freeze-thaw step, incubation with assay mix: HRP, 4-chloro-1-naphtol, substrate, NADP$^+$, buffer, 1 % agarose.

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